(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 300 469 A1

(12) EUROPEAN PATENT APPLICATION

(43) Date of publication:
09.04.2003 Bulletin 2003/15

(51) Int Cl.^{7}: **C12N 15/53**, C12N 15/82, C12N 9/02

(21) Application number: **02256996.6**

(22) Date of filing: **04.10.2002**

(84) Designated Contracting States:
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR IE IT LI LU MC NL PT SE SK TR
Designated Extension States:
AL LT LV MK RO SI

(30) Priority: **05.10.2001 JP 2001309824**

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(54) Expression of laccase from basidiomyctes in transgenic plants

(57) A plant which comprises a heterologous DNA encoding a phenoloxidase, wherein the said heterologous DNA is capable of being expressed in the said plant. A method of producing phenoloxidase wherein the plant is water-cultured. Further, a method of decomposing and removing hazardous chemical substances wherein the plant is cultivated in an environment contaminated with the hazardous chemical substances.

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Description

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] The present invention relates to a plant in which a phenoloxidase gene is introduced, a method of producing phenoloxidase using the plant, and a method of decomposing and removing hazardous chemical substances using the plant.

Description of the Related Art

[0002] Phenoloxidase is an enzyme which converts phenols to o-quinone or p-quinone by oxidization, and laccase is a representative example. Laccase is widely present in animals and plants, and fungi, and in recent years laccase could be obtained as an extracellular enzyme of a microbial cell having the ability to produce laccase. Laccase as an extracellular enzyme is produced and purified by purifying a culture filtrate of cells capable of producing the enzyme, or by conducting genetic recombination of laccase genes into hosts e.g. fungi such as Aspergillus genus, culture of the transformant in a liquid medium, and collection of laccase from the culture solution (YAVER, D.S. et al. (1996) Appl. Environ. Microbiol. 62: 834-841, Berka, R. M. et al. (1997) Appl. Environ. Microbiol. 63: 3151-3157). However, in producing laccase using microorganisms capable of producing laccase, particularly the above recombinant, the production efficiency is enhanced, but in the meantime a problem arises that culture and purification etc. are costly. Therefore, although there have been many reports on the usefulness of laccase in wastewater treatment (e.g. coagulative precipitation treatment of organochlorine compounds in pulp bleaching drainage), decomposition of hazardous chemical substances (e.g. decomposition of chlorinated phenols) (Roy-Arcand, L and Archibald, F. S. (1991) Enzyme Microbial Technol. 13: 194-203, Ricotta, A. et al. (1996) Bull Environ. Contam. Toxicol. 57: 560-567, Johannes, C. et al. (1996) Appl. Microbiol Biotechnol. 46: 313-317, Hoff, T. H. O. M. et al. (1985) Appl. Environ. Microbiol. 49: 1040-1045, Chivukula, M. U. R. A. and Renganathhan, V. (1995) Appl. Environ. Microbiol. 61: 4374-4377, Bollag J. -M. et al. (1988) Appl. Environ. Microbiol. 54: 3086-3091, Amitai, G. et al. (1998) FEBS Lett 438: 195-200), production of artificial lacquer paints, turbidity prevention for beverages, clinical analysis, etc., in fact, laccase has not come into practical use. Thus, laccase is brought to a commercial stage only for washing and decoloring denim, which is considered to have high added value.

[0003] Accordingly, there is a demand in many fields for a simple production method of phenoloxidase, in particular laccase, which has high production efficiency and desirable cost performance. Though there have been confirmed many patent applications or documents on production methods of phenoloxidase, e.g. laccase (Japanese Patent Application Laid-Open (kokai) No. 9-56378, Japanese Patent Application Laid-Open (kohyo) No. 9-503126, Japanese Patent Application Laid-Open (kohyo) No. 9-505481, YAVER, D. S. et al. (1996) Appl. Environ. Micorbiol. 62: 834-841, Berka, R. M. et al. (1997) Appl. Environ. Microbiol. 63: 3151-3157, etc.), a method which is satisfactory from a practical point of view has not yet been found.

[0004] On the other hand, physicochemical treatments are under development as treatment technologies for cases wherein hazardous chcmical substances such as PCB, BHC and DDT which are produced as industrial chemical substances, or dioxins which are unintentional products, are stocked at high concentrations or accumulated in an environment. For example, photochemical decomposition, supercritical decomposition, solvent extraction decomposition, catalytic oxidization, vapor phase hydrogenation reduction, melt combustion, heat treatment in reducing atmosphere and glassification treatment are under validation testing. However, these physicochemical methods are not practical, from a viewpoint of cost-effectiveness, for hazardous chemical substances accumulated at low levels in an environment such as in the soil or in rivers, and moreover in situ treatment methods for these substances are required. These hazardous chemical substances diffused over a wide area, even though their concentrations are low, have sufficient concentration levels for endocrine disruption. As a means to overcome this problem, bio-remediation has been conducted with the use of microorganisms that strongly decompose hazardous chemical substances, but such a decontamination method by microorganisms still has drawbacks. That is, the inoculation of the microorganisms and application of nutrient sources needs to be conducted in order to maintain dominance of such microorganisms over a long period, and this becomes more difficult as the contaminated area expands.

[0005] From this perspective, in recent years, attempts at decontamination have been made by phyto-remediation (restoration of the environment by plants) which utilizes plants. Plants can be grown independently taking nourishment from the sun, water, and inorganic ions, and can be cultivated extensively by controlling their seeds. Because of this, they have attracted attention as a sustainable environmental decontamination method.

[0006] The phyto-remediation that has been examined includes use of detoxification mechanisms or transpiration ability which plants inherently possess. Further, attempts to strengthen the environmental decontamination function of

plants have recently made by introducing microorganism-derived genes. However, environmental remediation by transformant plants that has been examined so far involves, in the case of, for example, agricultural chemicals, heavy metals, or the like, transportation and accumulation of these substances to and in cell fractions. Therefore, when the plants die, the accumulated environmental contaminants are released again into the environment and thus this does not lead to a fundamental solution for decontamination. Furthermore, in the case where the hazardous chemical substances are dioxins or PCB, it is predictable that readily degradable substances are decomposed while difficult-to-degrade and highly toxic substances are condensed and accumulated. Thus, there is no other choice to consider that conventional phytoremediation is insufficient.

[0007] Against this background, attempts to decompose hazardous chemical substances directly in plant cells using transformant plants into which an enzyme gene for decomposing hazardous chemical substance derived from microorganism are introduced, have been made with respect to 2,4,6-trichlorophenol (Japan Society for Bioscience, Biotechnology, and Agrochemistry, Abstracts for the Annual Meeting, p164, 1998) or γ-hexacyclohexane (Japan Society for Bioscience, Biotechnology, and Agrochemistry, Abstracts for the Annual Meeting, p89, 1997).

[0008] Incidentally, it has been clarified that laccase can decompose various chemical substances which are not readily degradable. Laccase can oxidatively decompose endocrine disrupting chemicals including chlorophenols, agricultural chemicals, polycyclic aroma hydrocarbons, alkyl phenol, aroma hydrocarbons, and nitro compounds.

[0009] Accordingly, when genes for phenoloxidase, e.g. laccase, are incorporated and plants which can express a function of the genes are prepared, a method of producing phenoloxidase at high yields and desirable cost levels can be established. Also, it is further possible to accomplish phyto-remediation which is useful for decomposing and removing hazardous chemical substances in the environment.

[0010] Although there have already been reports (Japanese Patent Application Laid-Open (kokai) Nos. 6-125782, 8-051986, etc.) on methods for obtaining transformant plants by introducing various foreign genes into plants, it is difficult to introduce active phenoloxidase into plants and enable stable secretion and production of the protein locally from roots thereof. Until now there have been no reports on preparation of such transformant plants, methods of producing phenoloxidase by such plants, and phyto-remediation utilizing such plants.

SUMMARY OF THE INVENTION

[0011] Under the above mentioned technical background, it is an object of the present invention to provide a plant which can secrete and produce phenoloxidase (hereinafter referred to as "protein of the present invention") e.g. laccase etc. from a root thereof, and a method of decomposing and removing hazardous chemical substances using the plant.

[0012] The present inventors have intensively made studies in order to solve the above problems. As a result, they have found that a plant into which a gene for phenoloxidase, e.g. laccase, is introduced can secrete and produce the enzyme from a root thereof and such plant enables decomposition and removal of hazardous chemical substances, thereby completing the present invention.

[0013] Namely, the present invention provides the following (1) to (10):

(1) A plant into which a DNA encoding phenoloxidase is introduced, the DNA being expressed therein.
(2) The plant according to (1), wherein the DNA is locally expressed in a root thereof.
(3) The plant according to (1) or (2), wherein the DNA encoding phenoloxidase is a DNA encoding laccase.
(4) The plant according to (3), wherein the laccase is a secretory laccase.
(5) The plant according to (3), wherein the laccase is derived from basidiomycete.
(6) The plant according to (5), wherein the basidiomycete belongs to the genus *Coriolus, Schizophyllum* or *Pleurotus*.
(7) A plant into which a DNA encoding the following protein (a) or (b) is introduced, the DNA being expressed therein:

(a) a protein having an amino acid sequence shown in SEQ ID NO:1; or
(b) a protein having an amino acid sequence comprising deletion, substitution, or addition of one or several amino acids relative to the amino acid sequence shown in SEQ ID NO:1, and which has laccase activity.

(8) The plant according to any of (1) to (7), wherein the plant is a seed plant.
(9) A method of producing phenoloxidase, comprising the steps of:

culturing the plant described in any one of (1) to (8) in a water culture solution; and
collecting phenoloxidase from the culture solution.

(10) A method of decomposing and removing hazardous chemical substances, comprising the step of:

cultivating the plant according to any one of (1) to (8) in an environment contaminated with a hazardous chemical substance.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014]

Fig. 1 shows a structure of plasmid pW35SfLac/pBI121.
Fig. 2 shows the results obtained by conducting isoelectric focusing and activity staining on laccase secreted in culture solutions.

DESCRIPTION OF SEQUENCE LISTING

[0015]

SEQ ID NO:3 is Primer LfXb.
SEQ ID NO:4 is Primer LBa.

[0016] This specification includes the contents as disclosed in the specification and/or drawings of Japanese Patent Application No. 2001-309824, which is a priority document of the present application.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0017] Hereinafter, the present invention will be described in detail.
[0018] A plant of the present invention is a plant which comprises a heterologous DNA encoding a phenoloxidase, wherein the said heterologous DNA is capable of being expressed in the said plant. Phenoloxidase is an enzyme which oxidises phonols thereby to convert them to o-quinone or p-quinone in the presence of oxygen. It includes monophenol oxidization enzymes such as tyrosinase, which act on not only polyphenols but also monophenols, and diphenol oxidization enzymes such as laccase, which act on only p-diphenols.
[0019] A heterologous DNA is one that is to be introduced into a plant or has been introduced into a plant, thus giving rise to a plant of the invention, i.e. a transgenic plant. A heterologous DNA may comprise sequences which originate from a species other than the plant comprising the heterologous DNA. Indeed, the heterologous DNA may comprise sequences which are not of plant origin. Such sequences may originate from, for example, a bacterium, a fungus or a different plant species. Alternatively or additionally, a heterologous DNA may comprise, or consist essentially of, sequences which originate from the species comprising the heterologous DNA. In the latter case, a plant of the invention may thus comprise more than one copy of a gene which occurs naturally in the species in question. Of course, a heterologous DNA may comprise sequences which originate from the species to be transformed and sequences which do not originate from the species to be transformed.
[0020] Among phenoloxidases, laccase is particularly preferable in terms of variety of applications, enzyme activity, ability to decompose and remove hazardous chemical substances, etc.
[0021] Laccase is present naturally and widely in animals, plants, and fungi. Though laccase from any source can be used for the present invention, laccase from various microorganism, e.g. basidiomycetes, ascomycetes, and hyphomycetes is preferable particularly in terms of operational ease in enzyme production (stability of enzyme, ability to reduce toxicity of hazardous chemical substances) etc. Among these, laccase obtained from basidiomycetes is particularly preferable. Examples of basidiomycetes include the microorganisms which belong to the genus *Coriolus, Schizophyllum* or *Pleurotus*, for example, *Coriolus versicolor, Schizophyllum commune,* and *Pleurotus ostreatus*.
[0022] Further, laccase which can be introduced into the plant of the present invention may be a secretory laccase. Herein, the phrase "secretory laccase" means laccase which is secreted outside of plant bodies via a signal sequence that is necessary to secrete proteins outside of plant bodies.
[0023] As the proteins of the present invention which can be introduced into plants, a protein having an amino acid sequence of SEQ ID NO:1 may be given. Further, the proteins of the present invention include not only a natural protein, having laccase activity, obtained from basidiomycete, but also a protein which may comprise substitution, deletion or addition of one or several amino acids relative to the natural protein as long as the protein does not lose the activity.
[0024] The polypeptide expressed by a plant of the invention may have an amino acid sequence comprising the deletion, substitution, or addition of one or more amino acids relative to the amino acid sequence shown in SEQ ID NO: 2 (so long as it demonstrates laccase activity). The sequence of SEQ ID NO: 2 may thus be modified by insertion, deletion, addition (for example N-terminal or C-terminal addition), or substitution of an amino acid with another amino acid. More than one such amino acid modification may be made, for example 1, at least 5, at least 10, at least 20, at

least 30, at least 50 up to about 70, 80, 100 or 150 such modifications. These types of modification may be combined to give a protein expressed by a plant of the invention. Amino acid substitutions will preferably be conservative substitutions, for example according to the following Table. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other.

ALIPHATIC	Non-polar	G A P
		I L V
	Polar-uncharged	C S T M
		N Q
	Polar-charged	D E
		KR
AROMATIC		H F W Y

[0025] Proteins having a shorter amino acid sequence than SEQ ID NO: 2, i.e. fragments, may also be expressed by a plant of the invention. For example, a peptide of from at least 100 amino acids or at least 200 amino acids up to about, 300, 400, or 500 amino acids in length may be expressed by a plant of the invention (so long as it demonstrates laccase activity).

[0026] In this sequence, a region comprising a peptide represented by the amino acid sequence of amino acid positions 1 to 21, functions as a signal sequence, and this signal sequence allows the protein of the present invention to stably and reliably be secreted outside of plant bodies. The signal sequence is a gene for secreting the protein and is functionally linked with N-terminus of laccase gene. Therefore, when it is desired to obtain from a transformant plant a secretory protein e.g. secretory laccase, it is preferable to avoid substitutions, deletions, and additions in the amino acid sequence of amino acid positions 1 to 21 in the amino acid sequence shown in SEQ ID NO:1.

[0027] Secretion and production can also be achieved by linking with phenoloxidase a known signal sequence, other than the signal sequence naturally existing in the laccase gene, which is represented by the amino acid sequence of the above amino acid positions 1 to 21. However, it is particularly preferable to employ the secretory laccase having the amino acid sequence, which comprises the signal sequence shown in SEQ ID NO:1, because it is possible to stably and reliably secrete laccase outside of plant bodies.

[0028] In order to isolate the DNA encoding the protein of the present invention which can be introduced into plants, gene cloning methods can be used. For example, there is a method wherein an enzyme is purified, an amino acid sequence is determined, and synthetic nucleotides are prepared based on the sequence to select the DNA from a gene library by hybridization. Moreover, there is also a method wherein primers used for PCR (Polymerase Chain Reaction) are prepared based on the known gene nucleotide sequence information without purifying the enzyme, to amplify and isolate a specific region or whole region of the gene by performing PCR.

[0029] The DNA encoding the protein of the present invention which can be introduced into plants, can be expressed by introducing it into plants with a suitable promoter or the like. The promoter includes a promoter which enables local expression. For example, cauliflower mosaic virus 35S promoter (CMV 35S-P) which enables strong expression in roots may be used. Further, in addition to this, known promoters which promote local expression in leaves or stems can be used, but operations for separation and purification of the proteins produced therefrom are complicated. Accordingly, it is particularly preferable to use a promoter which enables strong expression in roots. It is noted herein that the phrase "local expression" means not only expression occurring only in a particular local part of a plant but also local expression where an expression in a particular part is much stronger compared with other parts. The level of expression being much stronger refers to a case wherein the expression of interest stronger by approximately 1/3 to 1/2 compared with expression of other parts. Specifically, when it is said that DNA "can be locally expressed in roots," this phrase means that although expression in leaves or stems may be observed, the expression in roots is more significantly remarkable than expressions in those parts.

[0030] Further, it is possible to introduce into a plant a promoter which enhances expression. As an example of the promoter, a non-translation region in (CMV 35S-up) upstream of the CMV 35S-P can be used.

[0031] Any terminator can be used as long as it functions in plant cells, for example, the terminator of a nopaline synthase gene can be used.

[0032] For DNA introduction into plants, chemical, physical and biological methods including electroporation, a method using a particle gun, and a method using Agrobacterium can be used to introduce DNA into a plant genome. Plant cells into which DNA is introduced, can be selected and redifferentiated by the use of a marker such as drug-resistant property of antibiotics and the like.

[0033] The plants usable in the present invention include any plant species as long as the redifferentiating method

from cells, tissues, or organs is established and the gene introduction system is constructed. As preferable plant species, seed plants can be exemplified. Although the seed plants can be either herby plants or woody plants, herby plants are preferable due to easiness in cultivation, and examples thereof include tobacco, rice, and turf grass.

[0034] Since the protein of the present invention is secreted from the roots of the transformant plants prepared as above, the protein of the present invention can be obtained with few impurities by culturing the plants in a water culture solution and collecting the protein from the culture solution. The water culture solution is not particularly limited as long as it enables the transformant plants to grow therein and secrete the protein of the present invention, and Murashige & Skoog medium, for example, can be used. The transformant plant of the present invention is planted in the water culture solution and cultured at temperatures suitable for its growth. The time period necessary for sufficient secretion of phenoloxidase from the transformant plants varies depending on plant species to be used. Thus, while observing secretion condition of the enzyme, an appropriate culture period may be determined. However, usually the period is approximately one week. The protein of the invention secreted in the water culture solution can be collected by ordinary separation and purification methods for enzymes. For example, the culture solution containing phenoloxidase is centrifuged and further concentrated using a ultrafilter. Furthermore, a construction in which 6 histidine residues are linked with N- or C-terminus of phenoloxidase, is prepared and allowed to be expressed, and this can simplify the purification of the enzyme by purification resin of immobilized metal affinity chromatography. The thus obtained enzyme can be used in the fields of wastewater treatment, production of artificial lacquer paints, turbidity prevention of beverages, clinical analysis, etc.

[0035] Moreover, the protein of the present invention has a function to decompose and remove hazardous chemical substances as described above. Therefore, hazardous chemical substances can be decomposed and removed in the rooting zone sustainably and independently by cultivating the transformant plant of the present invention in environments contaminated with hazardous chemical substances. Further, it is possible to decompose and remove hazardous chemical substances by spraying the protein collected from the transformant plant of the present invention over the environments contaminated with hazardous chemical substances. As used herein, the term "hazardous chemical substances" means substances exhibiting toxicity or endocrine disruption to human bodies, and substances which can be decomposed or the toxicity of which can be reduced by the phenoloxidase, in particular laccase. To be more specific, examples thereof include chlorophenols, agricultural chemicals, polycyclic aromatic hydrocarbons, alkylphenol, aromatic hydrocarbons, and nitro compounds. Additionally, the term "environment" means, for example, soils, lakes, rivers and the like.

EXAMPLES

[Example 1] Cloning of laccase cDNA of *Coriolus versicolor*

[0036] In order to isolate cDNA encoding laccase, a nucleotide sequence of upstream and downstream regions of the region encoding laccase, that is 20 nucleotides from the initial codon and 22 nucleotides from the stop codon, was selected from nucleotide sequences of laccase genomes of *Coriolus versicolor* IFO 30340, which is registered under DDBJ accession No. D13372. Then, an oligonucleotide was chemically synthesized.

[0037] PCR was conducted using the above prepared oligonucleotide as primers and cDNA of *Coriolus versicolor* as a template. The reaction conditions for PCR were 25 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 90 seconds at 72°C. Pyrobest DNA polymerase (Takara Shuzo Co., Ltd.) was used for the reaction. A part of the reaction solution was subjected to 1.5 % agarose gel electrophoresis for separation, and then amplified products of DNA having the expected size were observed. Fragments of the amplified products were excised from agarose and extracted, and then subjected to sequencing, thereby obtaining full-length cDNA indicated in SEQ ID NO:1. Further, an amino acid sequence corresponding thereto is shown in SEQ ID NO:2.

[Example 2] Construction of a vector and introduction thereof into a plant

[0038] In order to obtain laccase cDNA into which a restriction enzyme site is introduced, primers were synthesized. Nucleotide sequences of respective primers are as follows.

LfXb: 5'-ttgtttctagatgtcgaggtttcactctct-3'

LBa: 5'-aattggatccttactggtcgctcgggtcgagcg-3'

[0039] Using the above synthesized DNA as primers and laccase cDNA of SEQ ID NO:1 as a template, PCR was conducted by Pyrobest DNA polymerase. The reaction conditions for PCR were 30 cycles of 10 seconds at 98°C, 30 seconds at 60°C, 80 seconds at 72°C. A fragment in which Xba I and Bam HI restriction sites were added to full-length laccase cDNA (SEQ ID NO:1), was obtained by LfXb and LBa primers, and was inserted into pCR2.1. The nucleotide sequence was confirmed by sequencing.

[0040] The cDNA fragment amplified above was excised from pCR2.1 and inserted into Xba I, Bam HI sites of plasmid pBI221 comprising CMV 35S-P which enables strong expression particularly in plant roots. That plasmid was named as pfLac/pBI221.

[0041] Further, CMV 35S-up was inserted into Hind III and Pst I sites upstream of CMV 35S-P of the plasmid pfLac/pBI221, thereby attempting to enhance expression efficiency of the laccase gene. The obtained plasmid was named as pW35SfLac/pBI221.

[0042] The plasmid pW35SfLac/pBI221 was digested with Hind III and Bam HI, and a fragment containing CMV 35S-up, CMV 35S-P and laccase gene was inserted into Hind III, Bam HI sites of pBI121. That plasmid was named as pW35SfLac/pBI121 (Fig. 1).

[0043] *Agrobacterium tumefaciens* LBA4404 strain carrying pW35SfLac/pBI121 was used to introduce a secretory laccase gene into tobacco SR1 strain, thereby obtaining a transformant plant. In addition, a transformant plant of tobacco SR1 strain was obtained using *Agrobacterium tumefaciens* LBA4404 strain carrying pBI121, which was used as control. Southern analysis and PCR analysis were performed with whole DNA derived from each individual thereby confirming that each gene had been introduced into each of these transformant plants.

[Example 3] Detection of laccase activity secreted from roots of transformant plants

[0044] Murashige & Skoog medium containing one-fifth of the prescribed amount of saccharose was prepared as a water culture solution. The transformant plants of tobacco SR1 strain, which had been prepared in Example 2 and grown to approximately 20 cm in length, were planted into the culture solution and cultivated at 28°C. After one-week of cultivation, 10 ml of the culture solution was collected, centrifuged at 12,000xg for 15 minutes, and concentrated to a concentration of 1/1,000 by a ultrafilter which eliminates molecules with a molecular weight of 10,000 or less. The protein concentration was then 10 μg/10 μl. This solution was developed by isoelectric focusing method and subjected to activity staining with 4-chloro-1-naphthol. The results are shown in Fig. 2. In the figure, numeral 1, numerals 2 to 12, and C1 to C2 represent the results for a crude enzyme solution of *Coriolus versicolor,* concentrates from the culture solution of transformant plants, and control plants (into which only pBI121 was introduced), respectively. As is clear from Fig. 2, a band around pI3.5 which is an isoelectric point for laccase (numerals 2 to 11 in Fig. 2) was clearly observed only from the culture solution of the transformant plant into which a laccase gene was introduced. Further, no band was observed in the culture solution, prepared under the same conditions, for the control plants into which only pBI121 was introduced (C1 and C2 in Fig. 2).

[0045] According to the results stated above, it was clarified that the transformant plant having a laccase gene introduced thereinto secretes from its roots laccase having activity. Therefore, it is confirmed that it is possible to enable a transformant plant to secrete laccase from its roots by introducing laccase genes into herby plants or woody plants and allowing expression in the transformant plants.

[0046] All publications, patents and patent applications cited herein are incorporated by reference in their entirety.

EFFECT OF THE INVENTION

[0047] A plant of the present invention into which a phenoloxidase gene is introduced enables efficient and low-cost production of phenoloxidase. Further, hazardous chemical substances can be decomposed and removed by cultivating this plant in an environment contaminated with hazardous chemical substances.

SEQUENCE LISTING

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<150> JP 2001-309824
<151> 2001-10-05

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Ser Thr Ser Ile His Trp His Gly Phe Phe Gln Lys Gly Thr Asn Trp

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Ser Phe Leu Tyr Asp Phe Gln Val Pro Asp Gln Ala Gly Thr Phe Trp

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Tyr His Ser His Leu Ser Thr Gln Tyr Cys Asp Gly Leu Arg Gly Pro

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Phe Val Val Tyr Glu Pro Asn Glu Pro Ala Ala Asp Leu Tyr Asp Val

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Ala Asn Val Gly Pro Ala Phe Pro Leu Gly Ala Asp Ala Thr Leu Ile

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Claims

1. A plant which comprises a heterologous DNA encoding a phenoloxidase, wherein the said heterologous DNA is capable of being expressed in the said plant.

2. The plant according to claim 1, wherein the DNA is locally expressed in a root thereof.

3. The plant according to claim 1 or 2, wherein the DNA encoding a phenoloxidase is a DNA encoding a laccase.

4. The plant according to claim 3, wherein the laccase is a secretory laccase.

5. The plant according to claim 3, wherein the laccase is derived from a basidiomycete.

6. The plant according to claim 5, wherein the basidiomycete belongs to the genus *Coriolus, Schizophyllum* or *Pleurotus*.

7. A plant which comprises a heterologous DNA encoding:

(a) a protein having the amino acid sequence shown in SEQ ID NO:2; or
(b) a protein having an amino acid sequence comprising the deletion, substitution, or addition of one or more amino acids relative to the amino acid sequence shown in SEQ ID NO:2, and which has laccase activity,

wherein the said heterologous DNA is capable of being expressed in the said plant.

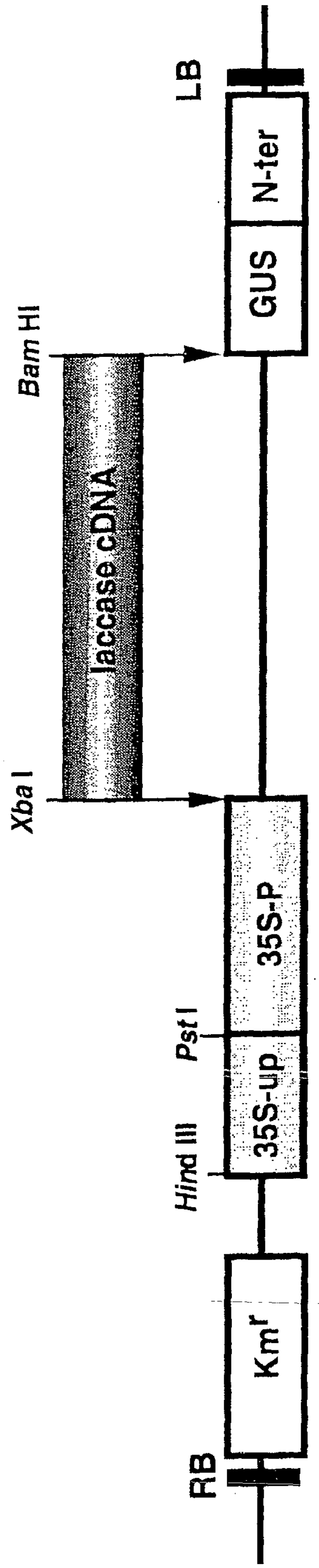
8. The plant according to any one of claims 1 to 7, wherein the plant is a seed plant.

9. A method of producing phenoloxidase, which method comprises:

culturing a plant according to any one of claims 1 to 8 in a water culture solution; and
collecting phenoloxidase from the culture solution.

10. A method of decomposing and removing a hazardous chemical substance from an environment, which method comprises:

cultivating a plant according to any one of claims 1 to 8 in the environment contaminated with a hazardous chemical substance.


LB
N-ter
GUS
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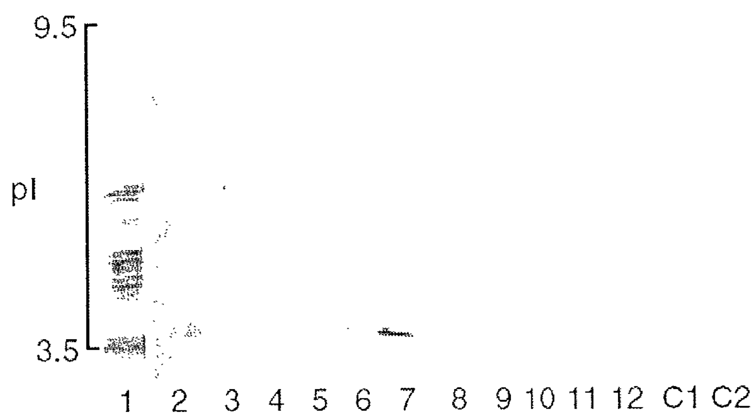
Pst I
35S-up
Hind III
Kmr
RB

FIG.1

FIG.2



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EUROPEAN SEARCH REPORT

Application Number
EP 02 25 6996

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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	WO 00 20615 A (HOWARD JOHN ;PRODIGENE INC (US); HOOD ELIZABETH (US); JILKA JOSEPH) 13 April 2000 (2000-04-13) * page 4, paragraph 2; claims 1,5,7,8,15,20,21; figures 1-4; examples 1-4 * * page 9, paragraph 3 - page 10, line 38 *	1-10	C12N15/53 C12N15/82 C12N9/02
A	LANG ELKE ET AL: "Production of ligninolytic enzymes by Pleurotus sp. and Dichomitus squalens in soil and lignocellulose substrate as influenced by soil microorganisms." FEMS MICROBIOLOGY LETTERS, vol. 167, no. 2, 15 October 1998 (1998-10-15), pages 239-244, XP002228445 ISSN: 0378-1097 * the whole document *		
			TECHNICAL FIELDS SEARCHED (Int.Cl.7)
A	IIMURA YOUSUKE ET AL: "Degradation and solubilization of 13C-, 14C-side chain labeled synthetic lignin (dehydrogenative polymerizate) by laccase III of Coriolus versicolor." BIOSCIENCE BIOTECHNOLOGY AND BIOCHEMISTRY, vol. 59, no. 5, 1995, pages 903-905, XP002228446 ISSN: 0916-8451 * the whole document *		C12N
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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	6 February 2003	Oderwald, H

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European Patent Office

EUROPEAN SEARCH REPORT

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DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
A	HATAMOTO OSAMU ET AL: "Cloning and expression of a cDNA encoding the laccase from Schizophyllum commune." BIOSCIENCE BIOTECHNOLOGY AND BIOCHEMISTRY, vol. 63, no. 1, January 1999 (1999-01), pages 58-64, XP002228447 ISSN: 0916-8451 * the whole document *		
A	GIARDINA PAOLA ET AL: "Cloning and Sequencing of a Laccase Gene from the Lignin-Degrading Basidiomycete Pleurotus ostreatus." APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 61, no. 6, 1995, pages 2408-2413, XP002228448 ISSN: 0099-2240 * the whole document *		
A	DATABASE EMBL 'Online! EBI Hinxton, UK; 10 September 1997 (1997-09-10) MIKUNI J AND IIMURA Y: "Coriolus versicolor CVL3 gene for laccase" Database accession no. D13372 XP002228449 * abstract *		TECHNICAL FIELDS SEARCHED (Int.Cl.7)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	6 February 2003	Oderwald, H

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06-02-2003

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		WO 0020615 A2	13-04-2000

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